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Research Article

The motility of breast cancer cells is stimulated by HMGB1/RAGE interaction but the truncated form lacking the C terminus has no effect

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Abstract

HMGB1/RAGE is identified as a ligand-receptor pair that plays an important role in tumorogenesis. HMGB1 and RAGE levels are higher in most human tumors and their overexpression is associated with tumor progression. The causes of breast cancer are still poorly understood. One reason might be the existence of subtypes within various cellular mechanisms as hormone-dependent and hormone -independent malignant processes. We investigated the effect of HMGB1 protein and its truncated form lacking the C terminus on the RAGE expression and cell motility of breast cancer cell lines; MCF7-noninvasive, MDA-MB-231-invasive and normal breast epithelial one MCF10. The results demonstrate that the effects of HMGB1 and HMGB1ΔC through RAGE association are observed exclusively for the hormone independent MDA-MB-231 cell line. The mobility of MDA-MB-231 cells was stimulated only by the full length HMGB1. Our results suggest that HMGB1/RAGE signaling should be considered as an essential process for the development of hormone independent breast cancers with great invasive potential. The truncated form plays the role of a blocking molecule that ”locks” the receptor and inactivates it. This makes the tailless molecule a promising therapeutic agent that competes for the biologically active HMGB1 ligand and prevents the downstream signaling through RAGE.

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Keywords

HMGB1-full length protein, HMGB1ΔC-truncated C-terminus, RAGE, breast cancer, cell motility

Introduction

Breast cancer is the most common malignancy among women (Siegel et al. 2015). The molecular mechanisms for the initiation, progression, and metastasis of breast cancer are not fully understood. During the last decade many data were published regarding High mobility group box-1 (HMGB1) protein as a putative regulator of the tumorigenesis, expansion, and invasion of cancer cells (Ellerman et al. 2007). HMGB1 was originally considered as a non-histone architectural chromosomal protein binding to distorted DNA structures (Pasheva et al. 1998). It is typically localized in the nucleus and functions as a nuclear cofactor in regulation of DNA repair, replication and transcription (Mitkova et al. 2005). The C-terminal negatively charged domain has modulatory functions either by its association with the HMG boxes A and B and thus regulating DNA binding (Knapp et al. 2004) or by accomplishing protein-protein interactions (Ge and Roeder 1994, Cato et al. 2008). However, HMGB1 can also be released into the extracellular matrix, where it has crucial role in carcinogenesis and inflammation (Tang et al. 2010). Once secreted, HMGB1 binds to its specific receptor RAGE (Receptor for Advanced Glycation End-products), and induces signal transduction. Recent studies have shown that for a large variety of human cancers, the levels of HMGB1 and its receptor RAGE are higher in tumor than in the normal surrounding epithelia (Tang et al. 2010). Moreover, these studies show that overexpression of HMGB1 is strongly correlated with tumor invasiveness and poor prognosis in cancer patients (Sparvero et al. 2009, Kostova et al. 2010). Several studies have suggested that HMGB1 could promote growth of breast cancer cells in vitro, but its exact role in patients with breast cancer is still unclear (Chalmers et al. 2013). The HMGB1/RAGE signaling was reported to be involved in several malignancies. The release of HMGB1 was considered as an inducement for acute antineoplastic inflammation, which was initiated against the tumor during chemotherapy (Apetoh et al. 2007, Kang et al. 2014). Thus, HMGB1 may serve as a biomarker of inflammation, as well as a prognostic marker for cancer progression. In a previous study we demonstrated that in all tested surgical pathology breast cancer specimens, the amount of HMGB1 protein was elevated. A well-defined perinuclear localization in the moderately differentiated breast carcinomas was observed whereas in the low differentiated cribriform breast carcinomas, the specific pattern was lost and the intense immune reaction was detected all over the nucleus. RAGE staining in ductal carcinoma samples displayed a characteristic granular pattern, but the signal was weak and detected in a limited number of cells. A higher level of RAGE was registered in the low differentiated cribriform breast carcinomas exhibiting diffuse cytoplasmic localization (Kostova et al. 2010). These data suggest that HMGB1/RAGE levels in breast cancer tissues significantly correlate with the differentiation grade and that HMGB1 may be clinically important in breast cancer. In this study we investigated the effect of HMGB1 protein and its truncated form lacking the C terminus on the expression of

endogenous RAGE in breast cancer cell lines (MCF7-noninvasive, MDA-MB-231-invasive) compared to normal breast epithelial one (MCF10). In this regard, we observed cell movement in treated cancer cell lines with certain concentrations of HMGB1 and its truncated form without the acidic C-tail for 24 hours. The mobility of the cancer cells was measured in the presence of the different HMGB1 protein forms in the extracellular medium.

Material and methods

Cell culture and DNA transfection

Human MDA-MB-231 and MCF-7 breast cancer cell lines as well as immortalized MCF-10A cell line were cultured according instruction of American Type Culture Collection (ATCC). Cells were kept in a 37°C incubator in 5% CO2. For plasmid transfection the cells were plated in 24-well dishes and transfected with 500 ng plasmid DNA using Lipofectamine 2000 (Thermo Fisher) according to the manufacturer's instructions.

Preparation of plasmid constructs and purification of recombinant HMGB1 proteins.

Full length, and truncated (ΔC) forms of HMGB1 were cloned in pET28a+ (Novagen) plasmid and expressed in modified Escherichia coli BL21 Poly Lys S as previously described (Elenkov et al. 2011).

RNA extraction and Real Time-PCR

Total RNAs were extracted with RNeasy kit (Qiagen) and treated with DNase I (Promega). 100 ng of total RNA were reverse-transcribed using oligo dT primers and SuperScript® First-Strand Synthesis System for RT-PCR (Thermo Fisher). Real-time PCRs were performed on a Rotor Gene 6000. Primers to assess transcript levels of human RAGE were: 5′- GTGCTGGTCCTCAGTCTGT and 5'-CTTCCCAGGAATCTGGTAGACAC, and for β-actin: 5'-AGAGCTACGAGCTGCCTGAC and 5'-AGCACTGTGTTGGCGTACAG. Expression data were normalized to the geometric mean of housekeeping gene cytoplasmic β-actin to control the variability in expression levels and were analyzed using the 2 -ΔΔCT method described by Livak and Schmittgen (2001).

Western blot analysis

Protein lysates (30–50 μg per sample) were separated on 12% SDS-polyacrylamide gels and transferred to Bio-Rad's 0,45 μm pore-size nitrocellulose membrane. Appropriate antibodies: anti-RAGE (AB9714, Merck Millipore 1:1000) and anti- β-actin (Thermo Fisher; 1:2000). Proteins were visualized using Li-cor Odyssey IR imaging system with appropriate IRDye-labeled secondary antibodies (Li-cor Biosciences).

Wound-healing assay

RAGEkd or control MDA-MB-321 were seeded (10,000 cells/well) in a 24-well culture dish. After attachment cells were grown in serum-free medium for 12 h and scratched using a

200-µl pipette tip (Rodriguez et al. 2005, Liang et al. 2007). After scratching the cells were washed and incubated in media with or without (control) HMGB1 recombinant proteins. Wound healing responses were photographed at 0, 4 and 24 hours. Each experiment was conducted in triplicate. AxioVision 4.8 software was used for the measurements.

Immunofluorescence

Immunofluorescence staining of cells was performed as previously described by Ugrinova et al. (2007), and visualized with secondary Alexa488 antibody and mounted with Fluoromount G (eBioscience). Antibody for the detection of RAGE (Merck-Millipore) were used at 1:500 dilutions. Images of cells were obtained with a Zeiss AxioVert 200M using a 63× objective.

esiRNAs preparation and RNA interference

Endoribonuclease prepared siRNAs (esiRNAs) targeting the coding regions of RAGE were synthesized as described (Yang et al. 2002, Kittler et al. 2005). Primers for targeted regions of RAGE (AGER) were selected using Riddle database (Kittler et al. 2007). The primers were: (5'-TCACTATAGGGAGAG GATCCAGGATGAGGGGATTT and 5'-TCACTATAGGGAGAC GCTACTGCTCCACCTTCTGG). For RNA interference 30 pmol of esiRNA and 2 µl of Lipofectamine 2000 were used per well in a 24 well plate (500 µl transfection volume). Knockdown of RAGE was assessed by Western blotting.

Statistical analysis

The data are presented as mean values ± SD of three to five independent experiments in duplicates (n= 6 –10) Student's t-test was used to compare means between groups. Statistical differences in wound healing and agarose spot assays were calculated by one-way single factor ANOVA test. P-values ≤0.05 were considered to indicate statistically significant results.

Results

Figs 1, 2, 3, 4

1. HMGB1 and HMGB1ΔC stimulate endogenous RAGE expression in breast cancer cells.

The endogenous expression of RAGE was measured at mRNA and protein level in the three breast cancer cell lines (Fig. 1**A**). The results clearly indicated that the amount of mRNA as well as the receptor were increased in the cancer cells. The increase was most prominent for MDA-MB-231 at protein level – almost twice. Immunofluorescence signal (Fig. 1**B**) corresponding to the receptor was diffuse in MCF7 cells while in MDA-MB-231 had a very well defined dot-like structure. Several studies have found that the expression of RAGE is regulated by its specific ligands glycated BSA and HMGB1 (Sharaf et al. 2015). To test if RAGE expression is affected by HMGB1 and HMGB1ΔC in our human cell lines,

they were incubated with rat **recombinant proteins** (10nM) for 4 hours and the mRNA and protein levels of RAGE were assessed. The homology between human and rat HMGB1 protein is very high (99%) and the RAGE binding sequence is completely conserved between the two species. As a control, cells were incubated with the same concentration of glycated BSA, a typical advanced glycation end product. In preliminary experiments we confirmed that glycated BSA indeed stimulates RAGE expression in comparison to unstimulated control (data not shown). The effect of HMGB1 and its truncated form (HMGB1ΔC) was stronger than the one obtained by glycated BSA (10nM) in the three cell lines (Fig. 2). In all cases the increase in the protein amount was slightly higher than the increase in mRNA. The overall increase was significantly better pronounced in the MDA-MB-231 invasive breast cancer cell line. (Fig. 2 compare panels **A** and **C**) Unstimulated endogenous expression of RAGE is shown in Fig. 1.

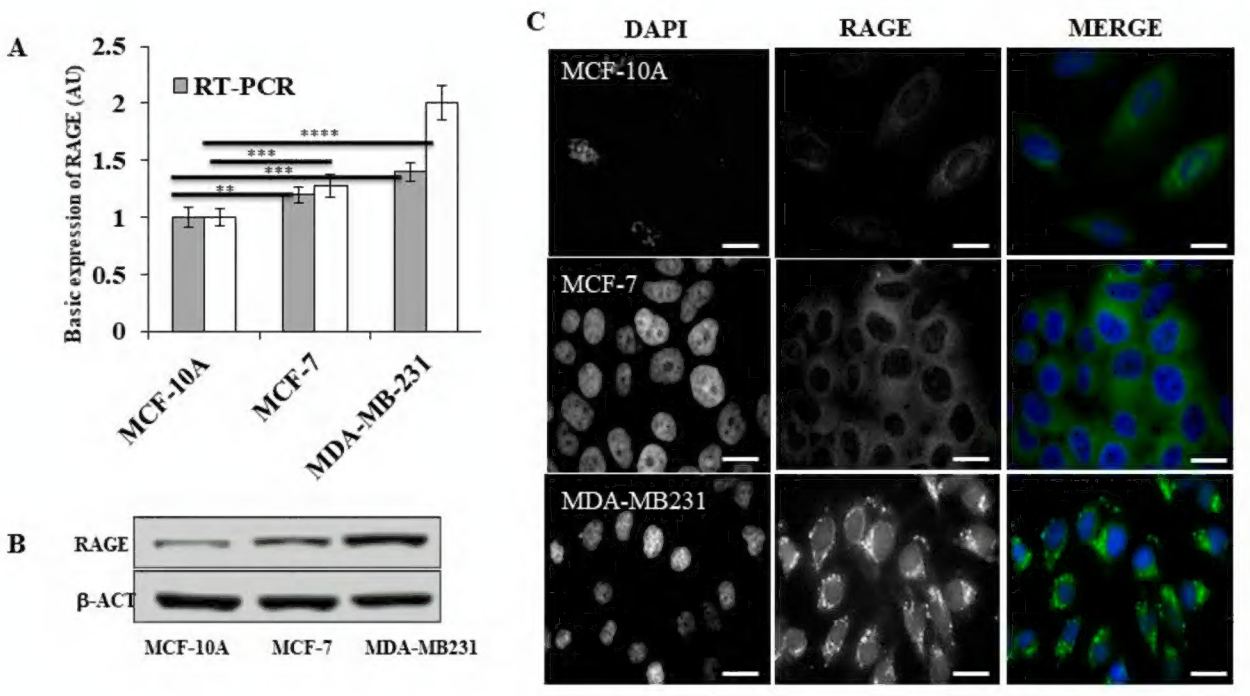

Figure 1.

Endogenous RAGE mRNA and protein expression in breast cancer cell lines. Panel A The expression of RAGE was examined at mRNA level by quantitative RT-PCR (gray) and at protein level by western blot analysis (white). AU stands for arbitrary units. The data are normalized to beta actin. The value of 1 corresponds to the respective levels of RAGE mRNA and protein in MCF-10A. The data represent the mean SD (*n* = 3). **Panel B** A representative western blot. **Panel C** Immunolocalization using an anti-RAGE antibody (1:500 dilution), DAPI-stained nucleus of MCF-7 and MDA-MB-231 cells. *Scale bars*, 20 μm.

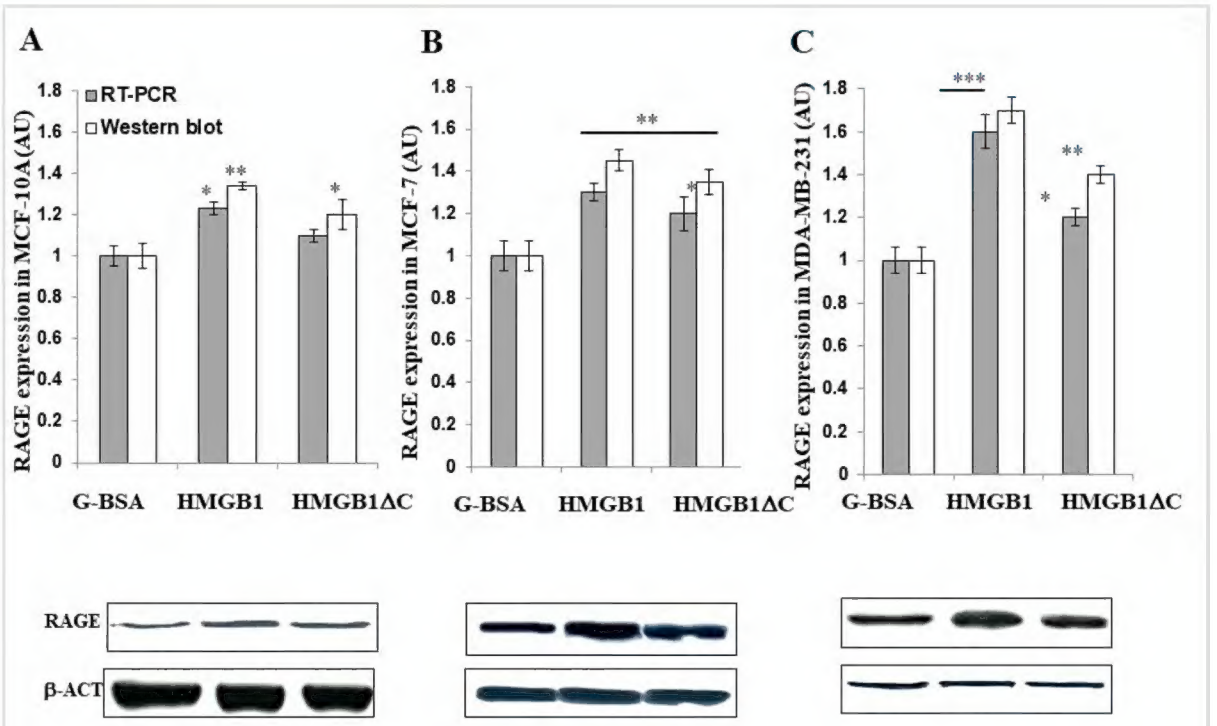

Figure 2.

The effect of HMGB1 and HMGB1ΔC on RAGE mRNA and protein expression in breast cancer cell lines: Panel A, MCF-10A; Panel B, MCF-7 and Panel C, MDA-MB-231. The expression of RAGE was examined by quantitative RT-PCR (gray) and by western blot analysis (white) 4 hours after incubation with 10nM final concentration of HMGB1 and HMGB1ΔC. The data are normalized to beta actin. The value of 1 corresponds to the levels of RAGE mRNA or protein in the respective cell line stimulated with glycated bovine serum albumin (10 nM G-BSA) as a model protein of AGEs. The data represent the mean SD (n = 5). The representative western blots are shown under the corresponding column charts. The statistical significance of the results was estimated by Student's t test - * 0.05 > P >0.01 and ** p value is 0.01 > P > 0.005 the stimulatory effect of HMGB1ΔC on MCF-10A cells did not differ statistically from the untreated control.

2. HMGB1 but not HMGB1ΔC increases MDA-MB-231 cell mobility.

HMGB1 protein is supposed to affect cell invasion, tumor growth and metastasis by high affinity binding to RAGE (Taguchi et al. 2000, Degryse et al. 2001). To study the effect of HMGB1 and HMGB1ΔC proteins on the mobility of MCF7 and MDA-MB-231 cells we did wound-healing experiments after treating the cancer cells with HMGB1 in/and without presence of its receptor RAGE.

To confirm the involvement of RAGE in the HMGB1 dependent cell invasion we silenced the receptor in MCF7 and MDA-MB-231 cancer cells using RNA interference with esiRNA. The decrease of RAGE abundance was up to 70% analyzed by Western blot analysis (Fig. 3). The cells were treated with 10nM recombinant HMGB1 and its truncated form HMGB1ΔC. Wound healing experiment was performed at two different cell lines. Cell motility was measured at 4 h and 24 h after treatment (Fig. 4).

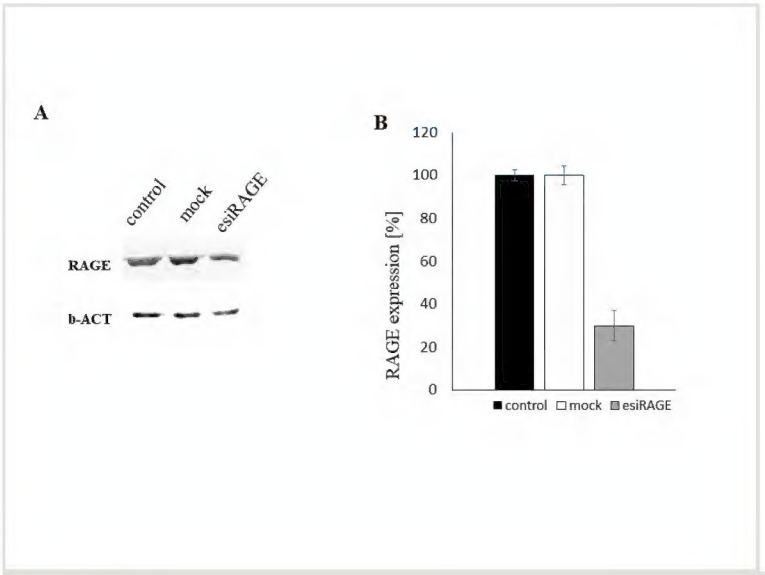

Figure 3.

Knock-down of RAGE protein expression by RNA interference. Panel A. The values of RAGE expression were normalized to beta actin and plotted. The graph shows about 70% down-regulated protein **(Panel B).**

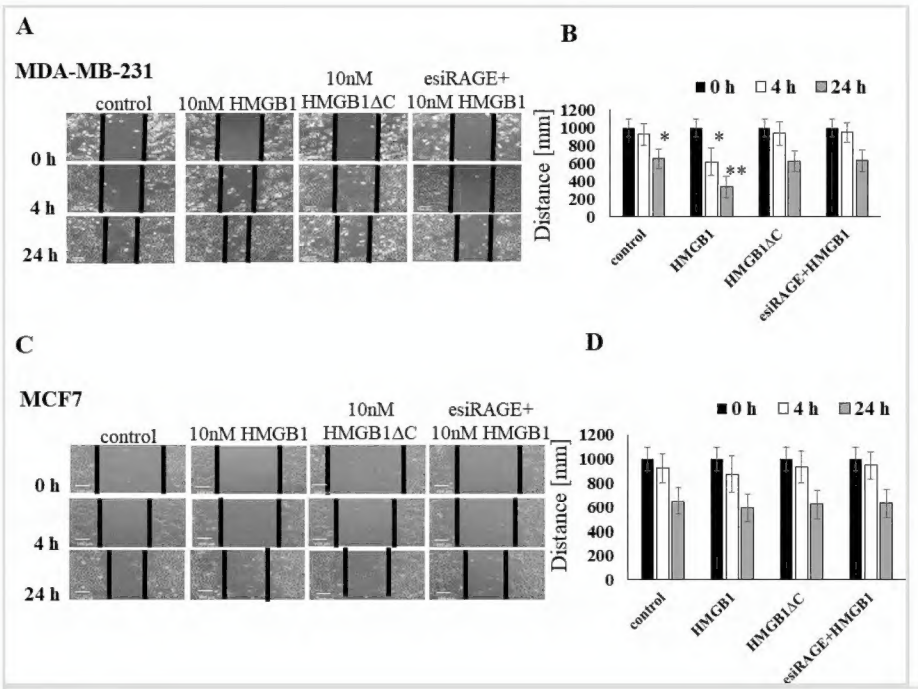

Figure 4.

The effect of HMGB1 and HMGB1ΔC recombinant proteins on the mobility of control and cancer cells with RAGE-expressed and RAGE silenced protein, measured by wound healing assay. Panel A. 90% confluent monolayers of MDA-MB-231 cells were scratch wounded. Images of wounded monolayer were taken at times 0 h, 4 h and 24 h. Cells are incubated with recHMGB1 and recHMGB1ΔC proteins (10 nM) with RAGE expressing cells and with silencing RAGE cells. The vertical lines indicate the wound edge. **Panel B and D.** Graphical representation of cell movement by measuring the distance between wound healing lines. The values represent the means ± SD (n=4). Statistical significance was calculated by Student's t test, and * P-values ≤0.05 were considered to indicate statistically significant results, **p-values ≤0.001. Bars indicate 20 μm. **Panel C.** 80% confluent monolayers of MCF 7 cells were scratch wounded. Images of wounded monolayer were taken at times 0 h, 4 h and 24 h. Cells are incubated with recHMGB1 and recHMGB1ΔC proteins (10 nM) with RAGE expressing cells and with silencing RAGE cells. The vertical lines indicate the wound edge and plotted the distance between wound healing lines.

As can be seen on Fig. 4, panel **A** and **B**, much more active cell movement was observed in MDA-MB 231 cells, treated with HMGB1 and no silencing RAGE. The truncated form of the protein (HMGB1ΔC) had almost no effect on cell motility compared to the control. The effect of HMGB1 and HMGB1ΔC on MCF7 was much less pronounced (Fig. 4, panel **C** and **D**). When RAGE is silenced and there is no interaction of extracellular HMGB1 with RAGE, the behavior of the treated cells is similar to that of the control. Wound healing assay in which we measured the gap filling clearly indicated that HMGB1 affected the mobility only in cells expressing RAGE. The RAGE-deficient cells behaved exactly as the control HMGB1-untreated cells (Fig. 4).

Discussion

HMGB1 is actively secreted by many cell types and high amounts of the protein are released to the extracellular space from necrotic cells and is a perfect candidate of a potential cellular stimulus. Extracellular HMGB1 is involved through RAGE in various human pathologies as diabetes, neuronal degeneration, inflammation (Schmidt et al. 2007, Yan et al. 2009), Alzheimer's disease (Yan et al. 2007), cardiovascular disease and cancers (Lin et al. 2012, Logsdon et al. 2007). There are accumulating data that RAGE acts as a signaling receptor for HMGB1 in several types of tumour cells (Huttunen et al. 1999, Rauvala et al. 2000, Taguchi et al. 2000). We studied the role of full length HMGB1 and HMGB1 lacking the C terminus for RAGE-mediated mobility and signaling in human breast cancer cell lines MCF-7 (ER +) and MDA-MB-231 (ER -). As we used recombinant proteins produced in prokaryotic expression system we were able to exclude the posible effect of postsynthetic modification of the protein on the studied processes. The presence of HMGB1 and HMGB1ΔC in the extracellular medium resulted in increase of endogenous RAGE protein and mRNA expression in all tested cell lines. Our data indicate that the stimulatory effect of HMGB1 and HMGB1ΔC was short-lived and more distinct for the full length HMGB1 in all cell lines. A specific granular structures were observed in MDA-MB-231 while in MCF7 the signal was diffuse. Similar observation was reported for RAGE immunolocalization in HEK293T cells (Zong et al. 2010). Several published data on the higher order structure of HMGB1 (Knapp et al. 2004, Stott et al. 2010) demonstrate that the flexible negatively charged C tail overlaps the HMG boxes A and B and thus probably masks the RAGE binding motif. The C-tail removal may facilitate the access of RAGE to HMGB1 region responsible for receptor/ligand association and explain the formation of a more stable complex of the tailless protein compared to full length HMGB1. Several studies have reported the extracellular role of HMGB1 in tumour cell migration (Parkkinen et al. 1993, Rauvala et al. 2000, Taguchi et al. 2000, Huttunen et al. 2002). The chemotaxis invasion of MCF7 and MDA-MB-231 was studied in the presence of full-length and truncated HMGB1 (Pasheva et al. 2018). The autors were shown that HMGB1ΔC formed a stable complex with RAGE and did not provoke any invasion in the agarose spot. On the contrary full-length HMGB1 increased substantially the mobility of MDA-MB-231 cancer cells, while the effect was much less expressed for MCF7 cells. Silencing of RAGE in MDA-MB-231 cells distinctly proved the involvement of RAGE in HMGB1 induced cell invasion as HMGB1 affected the cell mobility only in breast cancer cells expressing RAGE.

Finally, the results showed that HMGB1 induced a migration of breast cancer cells and the effect was RAGE-dependent. Some authors (Liu et al. 2019) have shown that when the HMGB1/RAGE bond is disrupted, the expression of PCNA and MMP9 decreases and the expression of p53 increases. They also showed that increasing the concentration of ethylpyruvate, which breaks the binding of the HMGB1 to RAGE-receptor, inhibits NF-kB and CTAT3 activation. Suppressing the HMGB1/RAGE axis leads to inhibition of cell growth and migration as well as induction of enhanced apoptosis of cancer cells. Moreover, Chen et al. (2014) demonstrate that HMGB1 activates RAGE signaling pathways and induces NF-κB activation to promote cellular proliferation, invasion, and metastasis, in hepatocelular cancer cell lines. A major player in the downstream signaling of HMGB1/RAGE complex is considered the nuclear factor NF-kB. The constitutive activation of NF-kB is thought to be involved in survival, invasion and metastasis in various types of cancers especially in hormone-independent breast cancer cells (Aggarwal and Gehlot 2009). Normally NF-kB is localized in the cytoplasm in its inactive form bound to the inhibitor IkBs and upon activation is relocalized in the nucleus (Baeuerle and Henkel 1994). This translocation is considered as a quick response to extracellular signal stimulation. Our previous data showed that only the full length HMGB1 induced NF-kB activation registered by its full translocation to the nucleus in the invasive hormone dependent MDA-MB-231 cancer cell line. Again the truncated HMGB1 did not reveal any signs of signal transduction in MDA-MB-231 (Pasheva et al. 2020). We are observed that in the hormone-dependent MCF7 cancer cells cell-movement was not influenced either by the full length or by the truncated HMGB1 protein and remained almost the same as a control cells. The different mechanism of action of HMGB1/RAGE axis in hormone-dependent and hormone-independent breast cancer cell lines needs further investigation.

Conclusions

Our results demonstrate that the effects of HMGB1 and its truncated form HMGB1ΔC through RAGE interaction affected mostly the hormone independent MDA-MB-231 cancer cells. The mobility of MDA-MB-231 cancer cells was stimulated only by the full length HMGB1. We suggest that the truncated form of HMGB1 lacking the C-tail is a putative blocking molecule that “locks” the receptor and inactivates it. This makes the tailless molecule a promising therapeutic agent which is able to compete for the original and biologically active HMGB1 ligand and prevents the downstream signaling through RAGE. In conclusion HMGB1/RAGE axis may become a potential target in cancer therapy strategy.

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